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Serotonergic Ligands Induce Spawning but not Oocyte Maturation in the Bivalve *Mactra chinensis* from Central Japan

PETER P. FONG[1,2], RYUSAKU DEGUCHI[1], AND KEIICHIRO KYOZUKA[1]

[1]*Asamushi Marine Biological Station, Asamushi, Aomori, 039-35, Japan, and* [2]*Department of Biology, Gettysburg College, Gettysburg, Pennsylvania 17325*

Abstract. We examined the spawning sensitivity to serotonin and serotonergic ligands in the Japanese bivalve *Mactra chinensis*. Spawning was induced by both injected and externally applied serotonin (5-hydroxytryptamine, 5-HT). The vertebrate 5-HT_2 receptor agonist alpha-methyl 5-HT and the selective $5HT_{1A}$ agonist 8-OH-DPAT were also effective at inducing spawning. However TFMPP (m-trifluoromethylphenylpiperazine, a verterbrate 5-HT_1 receptor agonist) and 1-methyl-chlorophenyl biguanide (a vertebrate 5-HT_3 agonist) were not effective spawning inducers. The 5-HT-induced spawning was blocked by mianserin (a vertebrate 5-HT_2 antagonist). The rank order of potency of the agonists was: 5-HT > alpha-methyl 5-HT > 8-OH-DPAT ≫ TFMPP > 1-methyl-chlorophenyl biguanide; these data support a growing body of literature invoking a mixed 5-HT_1/5-HT_2 pharmacological profile for serotonin receptors mediating reproductive processes in bivalves. However, neither 5-HT nor 8-OH-DPAT induced germinal vesicle breakdown (GVBD) in *Mactra* oocytes. Sperm induced GVBD in a high percentage of oocytes. This is the first report of a bivalve in which spawning, but not GVBD, can be induced by 5-HT. This result might be expected because *Mactra* spawns germinal vesicle oocytes that normally undergo GVBD upon fertilization, but is in contrast to the case of the closely related *Spisula* spp. in which serotonin induces both processes. The ability of 5-HT to induce spawning but not GVBD makes *Mactra chinensis* a model organism for studying spawning and meiotic mechanisms in bivalves.

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Introduction

The biogenic monoamine serotonin (5-hydroxytryptamine; 5-HT) is widely recognized as having salient neurohormonal effects on reproductive processes in bivalves including gamete release from gonad fragments (Matsutani and Nomura, 1982), spawning (Hirai *et al.*, 1988; Ram *et al.*, 1993), sperm reactivation (Kadam and Koide, 1990), parturition (Fong and Warner, 1995), and oocyte maturation (Kadam and Koide, 1989; Krantic *et al.*, 1991; Fong *et al.*, 1994; Gobet *et al.*, 1994; Deguchi and Osanai, 1995). Bivalves in the family Mactridae, especially *Spisula* spp., have provided especially good material for the study of the effect of serotonin on reproductive processes. Both spawning of prophase-I oocytes and the reinitiation of meiosis can be induced by 5-HT in *S. solidissima* and *S. sachalinensis* (Hirai *et al.*, 1988). Kadam and Koide (1989) tested various serotonergic ligands on oocyte maturation in *S. solidissima* and found that 8-OH-DPAT, a vertebrate $5\text{-}HT_{1A}$ agonist, significantly induced GVBD. Later Kadam *et al.* (1990) reported that both 8-OH-DPAT and alpha-methyl 5-HT, a 5-HT_2 agonist, stimulated uptake of $^{45}Ca^{2+}$. This uptake could be blocked by mianserin, a 5-HT_2 antagonist. More thorough pharmacological investigations of 5-HT-induced oocyte maturation in *S. solidissima* have been reported by Krantic *et al.* (1991).

Mactra chinensis and *Spisula* spp. are closedly related species in the bivalve family Mactridae. *M. chinensis* supports an important bivalve fishery in Japan (Sakurai *et al.*, 1992; Sakurai, 1993, 1994) and Korea (Chung *et al.*, 1987). As such, information on the physiological mechanisms regulating spawning and oocyte maturation in this species is important not only for aquacultural

purposes, but also to compare reproductive mechanisms in closely related species. We tested various serotonin analogs for effects on spawning and oocyte maturation in *M. chinensis*. Our experiments revealed that, as in *Spisula*, 5-HT and related compounds are potent inducers of spawning, but unlike *Spisula*, serotonergic compounds do not cause significant GVBD in *Mactra*.

Materials and Methods

Animals

Mature *Mactra chinensis* (4–6 cm shell length) were collected in May 1995 by fishermen in Tokyo Bay, Chiba Prefecture, and immediately shipped to the Asamushi Marine Biological Station. Animals were maintained in running seawater until used. All experiments were performed from 23 to 30 June 1995.

Spawning and GVBD assays

To induce spawning, animals were injected in the foot with 0.4 ml of 5-HT, 5-HT ligands, or natural seawater (SW) as a control. Immediately after injection, animals were placed in plastic containers containing 200 ml of SW, and observed for evidence of spawning. All spawning experiments lasted one hour, after which all nonspawners were injected with 5-HT (determined to be a good inducer of spawning after initial experiment) as a test for their ability to spawn.

The effect of mianserin, a vertebrate 5-HT_2 antagonist that inhibits a number of reproductive processes in marine and freshwater bivalves, was tested as follows. Various concentrations of antagonist were injected into the foot for 15–20 min before a subsequent injection of 5-HT combined with mianserin at the same concentration as in the first injection. Responses were monitored for 1 h after the second injection.

For the analysis of GVBD, prophase-1 oocytes were dissected from ovaries or, on one occasion, obtained by a natural spawning. In both cases, released oocytes had large germinal vesicles that were clearly visible (Deguchi and Osanai, 1994). GV oocytes were pipetted into wells of a 24-well culture plate and subjected to various serotonergic agents, SW, or sperm from dissected testes. After 30–40 min, 70–211 oocytes were observed for germinal vesicle breakdown and the percent of the oocytes that had undergone GVBD was calculated.

Data for comparisons of percent spawning between treatments and controls were analyzed with Fisher's Exact Test (Zar, 1984).

Serotonin and mianserin were purchased from Sigma Chemical Co. (St. Louis, Missouri). All other ligands were obtained from either Research Biochemicals Inc. (Natick, Massachusetts) or Funakoshi Co. (Japan).

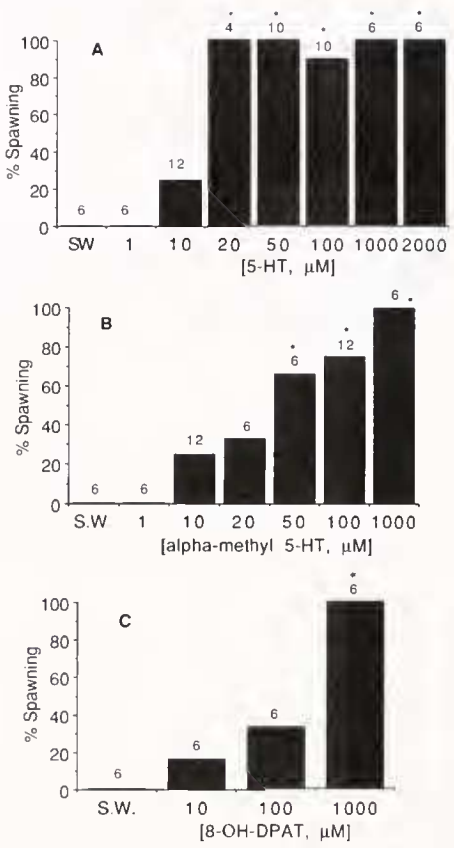

Figure 1. Percent spawning of *Mactra chinensis* after injection of serotonergic compounds or vehicle control (seawater, SW). (A) Serotonin (5-HT). (B) Alpha-methyl-5-HT. (C) 8-OH-DPAT. Numbers of clams tested are given above bars. *: $P < 0.05$ compared to control (SW). Data from males ($n = 62$) and females ($n = 60$) have been combined as there was no significant difference in responses between sexes.

Results

Both male and female *Mactra chinensis* responded strongly to injected 5-HT. Compared with the seawater control, 5-HT concentrations from 20 μM to 2 mM significantly induced spawning in 90–100% of clams tested (Fisher's Exact Test; $P < 0.004$–0.0001; Fig. 1A). In all cases, spawning took place in <3 min, and in 1 mM 5-HT, spawning was extremely rapid (mean time to spawning = 46.4 s for males; 43.0 s for females). Spawned oocytes had germinal vesicles and were fertilizable with spawned sperm. The 5-HT_2 agonist alpha-methyl 5-HT also significantly induced spawning in both males and females at concentrations from 50 μM to 1 mM (Fisher's Exact Test, $P < 0.03$–0.001; Fig. 1B). The 5-HT_{1A} agonist 8-OH-DPAT also stimulated

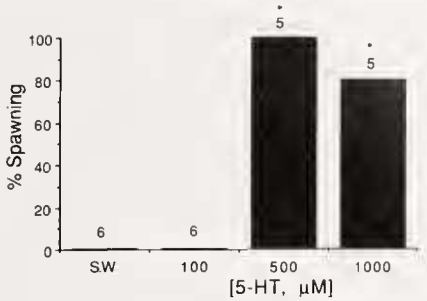

Figure 2. Percent spawning of *Mactra chinensis* after external application of different concentrations of 5-HT or seawater (SW). Numbers of clams tested are given above bars. *: $P < 0.05$ compared to control (SW).

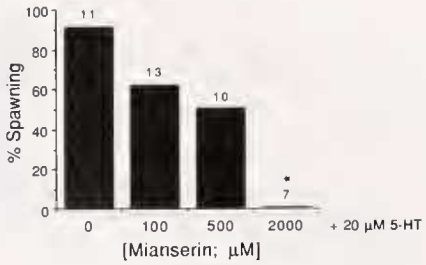

Figure 4. Effect of various concentrations of mianserin on spawning induced by 20 μM 5-HT in *Mactra chinensis*. Clams were first injected with mianserin, then after 15–20 min, a second injection of 5-HT was given. Numbers of clams tested are given above bars. *: $P <$ 0.05 compared to "no mianserin" control.

spawning, but was not as potent as either 5-HT or alpha-methyl 5-HT; concentrations as high as 1 mM were required to produce significantly more spawning than controls (Fisher's Exact Test, $P < 0.001$; Fig. 1C). None of the 18 SW-injected controls spawned in the above series of experiments. Furthermore, neither the 5-HT_1 agonist TFMPP nor the 5-HT_3 agonist 1-methyl-chlorophenyl biguanide (1-m-c-b) were effective at inducing spawning. In TFMPP, only 4% (1 of 25) of the clams spawned, and in 1-m-c-b, none of the six clams tested spawned. External application of 5-HT (500 μM and 1 mM) was sufficient to induce a high percentage of spawning (Fisher's Exact Test, $P < 0.002$–0.001; Fig. 2).

Although both 5-HT and alpha-methyl 5-HT were potent agonists, *Mactra* responded to 5-HT more rapidly. At 50 μM and 100 μM, significant (t-tests; $P < 0.05$–0.06) differences in time to spawning exist between clams in 5-HT and alpha-methyl 5-HT (Fig. 3).

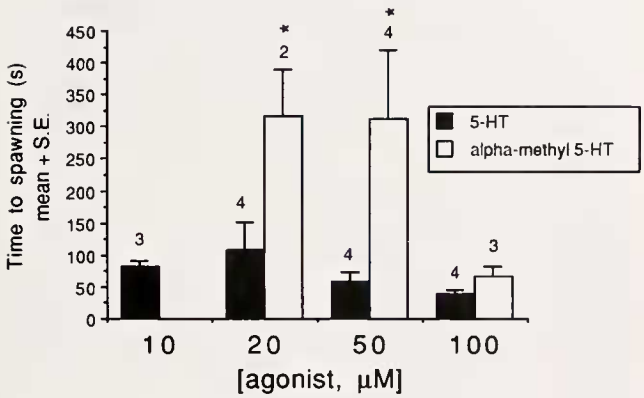

Figure 3. Time to spawning (mean + S.E.) of *Mactra chinensis* after injection of various concentrations of either 5-HT (filled bars) or alpha-methyl-5-HT (open bars). Numbers of clams that spawned are given above error bars. *: $P < 0.05$.

The specific 5-HT_2 antagonist mianserin effectively blocked, in a dose-dependent manner, the spawning induced by 5-HT(20 μM) (Fig. 4). Pre-treatment of clams with mianserin (2 mM) significantly blocked spawning produced by the injection of 5-HT (Fisher's Exact Test, $P < 0.002$). Moreover, even though clams pre-treated with 500 μM mianserin spawned after injection with 5-HT, some groups of clams took longer, on average, to spawn than control clams. Latency to spawning of clams pre-treated with 500 μM mianserin and then injected with 5-HT took significantly longer to spawn than the 20 μM 5-

Table I

Summary of experiments with Mactra chinensis *on the blockage of serotonin-induced spawning by mianserin, a 5-HT_2 antagonist*

1st injection	2nd injection	# Spawned/ total	Time (s) to spawning. Mean + (SEM)
20 μm 5-HT		9/10	75.7 (7.6)
1 mM 5-HT		8/8	37.0 (4.2)
100 μm mianserin		2/8	900.0 (12.5)
100 μm mianserin	20 μm 5-HT + 100 μm mianserin	8/13	340.8 (210)
500 μm mianserin		0/6	
500 μm mianserin	20 μm 5-HT + 500 μm mianserin	5/10	125.8 (18.9)
500 μm mianserin	1 mM 5-HT + 500 μm mianserin	6/6	83.3 (27.1)
2 mM mianserin		0/6	
2 mM mianserin	20 μm 5-HT + 2 mM mianserin	0/7	
2 mM mianserin	1 mM 5-HT + 2 mM mianserin	6/6	110.8 (73.9)

In cases where two injections were made, the second injection was given 15–20 min after the first injection. 5-HT: Serotonin.

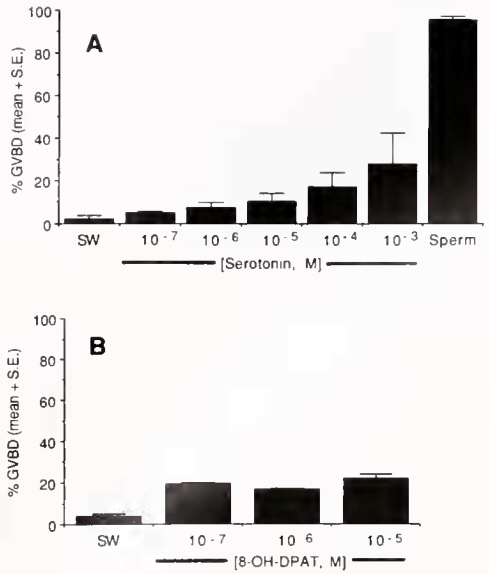

Figure 5. Percent GVBD (mean + S.E.) in *Mactra* oocytes treated with (A) 5-HT, or sperm as a positive control ($n = 6$ females) and (B) 8-OH-DPAT ($n = 3$ females). SW = seawater.

HT control (*t*-test, $t = 2.9$, $P < 0.01$), and the 1 m*M* 5-HT control (*t*-test, $t = 1.9$, $P < 0.07$; Table I).

In contrast to spawning, oocyte maturation as measured by GVBD was not significantly induced by 5-HT or its ligands. Neither 5-HT nor 8-OH-DPAT induced GVBD in a high percentage of oocytes, whereas sperm induced GVBD in over 95% of the oocytes (Fig. 5A, B).

Discussion

Injection of 5-HT, alpha-methyl 5-HT, a 5-HT_2 agonist, and 8-OH-DPAT, a 5-HT_{1A} agonist all produced significant dose-dependent induction of spawning in *Mactra chinensis*. In addition, external application of 5-HT also induced spawning. However, the 5-HT_1 receptor agonist TFMPP and the 5-HT_3 receptor agonist 1-m-chlorophenyl biguanide had little or no effect on spawning. Serotonin was effective at inducing spawning at lower concentrations than both alpha-methyl 5-HT and 8-OH-DPAT, and clams injected with 5-HT spawned significantly faster than those injected with alpha-methyl 5-HT. Therefore, the rank order of potency for agonists was 5-HT > alpha-methyl 5-HT > 8-OH-DPAT ⩾ TFMPP > 1-methyl-chlorophenyl biguanide. These experiments support a growing body of literature indicating that 5-HT receptors mediating reproductive processes in bivalves show a mixed $5\text{-HT}_1/5\text{-HT}_2$ pharmacological profile, unlike any described for vertebrates (*e.g.*, Kadam *et al.*, 1991; Krantic *et al.*, 1991; Fong *et al.*, 1993; Gobet *et al.*, 1994).

In bivalve molluscs, all oocytes are initially arrested at prophase-I within the ovary. At the time of spawning, oocytes are released either at prophase-I or metaphase-I. In species such as *Mytilus edulis*, *Dreissena polymorpha*, and *Ruditapes philippinarum*, prophase-I-arrested oocytes reinitiate meiosis (including GVBD) under the influence of neurohormones such as 5-HT. Such oocytes are spawned at metaphase-I where they remain until fertilization. By contrast, species such as *Spisula solidissima*, *Barnea candida*, and *Mactra chinensis* spawn prophase-I arrested oocytes which, in nature, only reinitiate meiosis after fertilization. In the oyster *Crassostrea gigas*, both prophase-I and metaphase-I oocytes can be fertilized, although in nature the latter occurs.

Oocytes from *Spisula* spp. have provided good material for the study of oocyte maturation and other cell cycle processes (Hirai *et al.*, 1988; Kadam and Koide, 1989; Kadam *et al.*, 1990; Krantic *et al.*, 1991; Dube, 1992). In *Spisula*, both spawning and GVBD can be induced by 5-HT and 5-HT agonists. However, considering that *Spisula* spawns prophase-I oocytes, the fact that 5-HT can induce GVBD seems puzzling in a functional sense. The reported 5-HT concentrations necessary to induce GVBD in spawned oocytes would not be present freely in seawater. Thus any effect of 5-HT or related agents on GVBD may simply mimic that of sperm. However, Juneja *et al.* (1993) found that lower concentrations of 5-HT enhanced the fertilizability of *Spisula* oocytes, indicating a 5-HT mediated process that might occur in the *Spisula* gonad during spawning. Although functional 5-HT receptors have been reported in *Spisula* oocyte plasma membranes (Kadam *et al.*, 1991), and were initially named 5-HT_5 (Krantic *et al.*, 1993), Guerrier *et al.* (1993) considered these receptors evolutionary vestiges from an ancestor with metaphase-I-arrested oocytes. In our experiments, we found that 5-HT and 5-HT ligands induce spawning, but do not induce GVBD in a large percentage of dissected or naturally spawned oocytes of *Mactra chinensis*. In contrast and as expected, sperm induced a high percentage of oocytes to undergo GVBD. Thus, within the animal, serotonin may be released internally by nerve terminals to bind to receptors to initiate spawning; but spawned oocytes, which never encounter high concentrations of 5-HT, are not sensitive to it. This is the first report of a bivalve in which 5-HT can induce spawning, but not GVBD. Because spawned GV oocytes are not responsive to 5-HT, GVBD can be examined without first sensitizing the oocytes to 5-HT. Thus, *Mactra chinensis* may be a model organism for studying spawning and oocyte maturation mechanisms in bivalves.

Although the importance of serotonin as a neurohormone controlling reproductive processes in bivalves has

Table II

Reproductive processes induced by serotonin and serotonergic ligands in selected bivalve species

Species (Family)	Reproductive process	Serotonergic ligand	Reference
Dreissena polymorpha (Dreissenidae)	Spawning	5-HT, 8-OH-DPAT, TFMPP, 1-NP	Fong *et al.*, 1993
	GVBD	5-HT, 8-OH-DPAT	Fong *et al.*, 1994
Ruditapes philippinarum (Veneridae)	Spawning	5-HT	Osanai and Kuraishi, 1988
	GVBD	5-HT, 8-OH-DPAT, TFMPP	Gobet *et al.*, 1994; Osanai and Kuraishi, 1988
Patinopecten yessoensis (Pectinidae)	Release of oocytes from ovarian fragments	5-HT	Matsutani and Nomura, 1982
Spisula solidissima (Mactridae)	Spawning	5-HT	Hirai *et al.*, 1988
	GVBD	5-HT	Hirai *et al.*, 1988
		8-OH-DPAT	Krantic *et al.*, 1991
		alpha-methyl 5-HT	Kadam *et al.*, 1991
	Sperm motility	5-HT, 8-OH-DPAT, 5-methoxytryptamine	Kadam and Koide, 1990
Crassostrea gigas (Ostreidae)	GVBD	5-HT	Osanai and Kuraishi, 1988
Hiatella flaccida (Hiatellidae)	GVBD	5-HT	Togo *et al.*, 1993
Potamocorbula amurensis (Corbulidae)	Spawning	5-HT, 8-OH-DPAT	Pers. obs.
Sphaerium transversum (Sphaeriidae)	Parturition	5-HT	Fong and Warner, 1995
		alpha-methyl 5-HT	unpub. data

5-HT = 5-Hydroxytryptamine, 8-OH-DPAT = 8-Hydroxydipropylaminotetralin hydrobromide, TFMPP = m-trifluoromethylphenylpiperazine, 1-NP = 1-(1-naphthyl)piperazine.

been well established, the number of species known to respond to this biogenic amine and similar compounds is still quite small and is restricted mainly to economically important species (Table II). Characterization of 5-HT receptors has been reported in only a few bivalve species such as *Dreissena polymorpha* (Fong *et al.*, 1993) and *Spisula solidissima* (Krantic *et al.*, 1993). In the gastropod *Lymnaea stagnalis*, a gene for a G-protein coupled 5-HT receptor (5-HTlym) was cloned and expressed in COS-7 cells (Sugamori *et al.*, 1993). To our knowledge, however, no bivalve serotonin receptors have been cloned and sequenced; hence we know little about the structure or evolutionary history of these proteins. These and further studies on the reproductive effects of 5-HT on a diverse array of bivalve species are required if the evolution of bivalve and molluscan 5-HT receptors is to be better understood.

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